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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: R&G Case 334 U.S. APPLICATION NO.

(If known, see 37 CFR 1.5): Unknown

INTERNATIONAL APPLICATION NO.: PCT/GB00/03182 INTERNATIONAL FILING DATE: August 16, 2000

PRIORITY DATE CLAIMED: August 19, 1999

TITLE OF INVENTION: HIGH DENSITY LIPOPROTEIN AGAINST ORGAN DYSFUNCTION FOLLOWING HAEMORRHAGIC SHOCK

APPLICANTS FOR DO/EO/US: (1) Gillian COCKERILL, (2) Norman MILLER, (3) Christoph THIEMERMANN and (4) Michelle MACDONALD

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    Formal Drawings (15 sheets)
    Title Page of WIPO Document WO 01/13939
    International Search Report
    Postal Card

FORM PTO-1390

U.S. APPLICATION NO. (if know, see 37 CFR 1.5): Unknown	INTERNATIONAL APPLICATION NO.: PCT/GB00/03182	ATTORNEY'S DOCKET NUMBER R&G Case 334

17. [X] The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00

International preliminary examination fee (37CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... $ 890.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... $ 740.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... $ 710.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ... $ 100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $890.00

Surcharge of $130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)). $

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	6 - 20 =	0	X $ 18.00	$
Ind. claims	2 - 3 =	0	X $ 84.00	$
MULTIPLE DEPENDENT CLAIMS (if applicable)			+ $280.00	$

TOTAL OF ABOVE CALCULATIONS = $890.00

Reduction of 1/2 for filing by small entity, if applicable. Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28). - $

SUBTOTAL = $890.00

Processing fee of $130.00 for furnishing the English translation later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). + $

TOTAL NATIONAL FEE = $890.00

Fee for recording assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property + $

TOTAL FEES ENCLOSED = $890.00

Amount to be refunded $

charged $

a. [X] A check in the amount of $890.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1382. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

IN DUPLICATE

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IN THE U.S. PATENT AND TRADEMARK OFFICE

February 15, 2002

Applicants: Gillian COCKERILL et al

For : HIGH DENSITY LIPOPROTEIN AGAINST ORGAN DYSFUNCTION FOLLOWING HAEMORRHAGIC SHOCK

PCT International Application No.: PCT/GB00/03182

PCT International Filing Date: August 16, 2000

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(if known, see 37 CFR 1.5): Unknown

Atty. Docket No.: R&G Case 334

Box PCT
Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT CANCELING CLAIMS

Sir:

Prior to calculation of the filing fee in the above-identified application, kindly enter the following:

IN THE CLAIMS

Please amend Claims 3-5 as shown below. A marked-up copy of the amended claims is enclosed herewith.

3. (Amended) Use according to claim 1 where the high density lipoprotein or derivative thereof is a peptide or protein derivative of the sequence of apo A-I or apo A-II, or a peptide or protein derivative functionally homologous to the active portions of apo A-I or apo A-II.

4. (Amended) Use according to claim 1 where the high density lipoprotein is reconstituted HDL.

5. (Amended) Use according to claim 1 where the high density lipoprotein is native HDL.

REMARKS

This amendment cancels claims to reduce the filing fee. Please enter this amendment before calculating the filing fee.

Respectfully submitted,

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Encl: Marked-Up Amended Claims 3-5

336.9804

MARKED-UP AMENDED CLAIMS 3-5

3. (Amended) Use according to claim 1~~ or 2~~ where the high density lipoprotein or derivative thereof is a peptide or protein derivative of the sequence of apo A-I or apo A-II, or a peptide or protein derivative functionally homologous to the active portions of apo A-I or apo A-II.

4. (Amended) Use according to claim 1~~ or 2~~ where the high density lipoprotein is reconstituted HDL.

5. (Amended) Use according to claim 1~~ or 2~~ where the high density lipoprotein is native HDL.

15/pRT>

HIGH DENSITY LIPOPROTEIN AGAINST ORGAN DYSFUNCTION FOLLOWING HAEMORRHAGIC SHOCK

The present invention relates to the manufacture of medicaments for protecting against organ damage following haemorrhagic shock, using high-density lipoproteins (HDLs) and derivatives thereof. In particular, it relates to manufacture of medicaments for treatment and prevention of end-stage organ failure following haemorrhagic shock.

Many victims of sudden physical injury (for example, traffic accident victims) die because of end-stage organ failure. In patients with this condition, biochemical and biological changes (such as haemodynamic changes and microthrombus formation) occur in the blood and organs (such as liver and kidneys) due to shock and blood loss; this is a different action to "endotoxic" shock which arises due to bacterial infection. If end-stage organ failure is not halted or prevented, it will lead to permanent organ damage and death of the patient. There is a need for a pharmaceutical agent which can be administered as soon as possible after the physical injury, preferably at the site of the accident in order to prevent end stage organ failure, and which can also be used subsequently while transporting the victim from the accident site to casualty/hospital, and while the physical wounds are being treated.

High-density lipoproteins (HDLs) form a range of lipoprotein particles found in normal serum. Mature HDL particles are present in the form of a globular structure containing proteins and lipids. Within the outer layer of these

particles are the more polar lipids, phospholipids and free cholesterol, all having charged groups pointing outwards towards the aqueous environment. The more hydrophobic lipids, such as esterified cholesterol and triglycerides, reside in the core of the particle. Newly formed, or nascent, HDL particles lack the lipid core and are discoidal in shape. Protein components are embedded in the outer layer. The main protein component is apolipoprotein A-I (apo A-I), with smaller amounts of apo A-II, apo A-IV, apo CIII, apo D, apo E and apo J. Various other proteins reside on the HDL particle, such as lecithin-cholesterol acetyl transferase, PAF acetylhydrolase and paraoxonase.

The binding of activated leukocytes to the endothelium is the earliest observable cellular event in a number of acute and chronic inflammatory diseases. This binding is mediated by the expression of adhesion molecules on the surface of the endothelial cells which bind to corresponding molecules of similar function on leukocytes. Recently we have shown that pre-treatment of endothelial cells, *in vitro*, with HDL was able to inhibit the cytokine-induced expression of these adhesion molecules (Cockerill GW, Rye K-A, Gamble JR, Vadas MA, Barter PJ. Arterioscler Thromb. Vasc. Biol. 1995, 15: 1987-1994 1995, Cockerill GW Reed S.Int.Rev.Cytol: A survey of cell biology 1999). In addition, we have recently shown that HDL can inhibit cytokine-induced adhesion molecule expression in an acute inflammatory model in the pig (Cockerill et al., submitted 1999). The antiflammatory effects of HDL have thus been demonstrated in these models where cells/animals are pre-treated with lipoprotein.

End-stage organ failure following haemorrhagic shock results from the adhesion of polymorphonuclear leukocytes (PMNs) to the endothelium following their activation caused by ischaemia and reperfusion injury. We have now found that administration of HDL or derivatives thereof prevents end-stage organ failure following haemorrhagic shock.

According to the present invention high density lipoprotein and/or a derivative thereof is used in the manufacture of a medicament for the prevention or treatment of organ dysfunction following haemorrhagic shock.

Preferably, the medicament is for the treatment of end-stage organ injury or failure.

We have shown that, following haemorrhagic shock, HDL is able to perturb the damaging effects when given after the initial hypovolaemia has occurred. Our work suggests that at physiological levels (both *in vitro* and *in vivo*), native HDL particles are active in inhibiting the expression of adhesion proteins on endothelial cells. Prevention of expression of adhesion proteins on endothelial cells prevents binding of PMNs to the endothlium; thus administration of HDL prevents end-stage organ failure.

The high density lipoprotein may be the component of HDL that inhibits adhesion to the endothelial cells and subsequent activation of leukocytes or a derivative, molecule, homologue, or mimic thereof.

The inhibiting effect is not only present in venous endothelial cells but also on arterial endothelial cells and is independent of the nature of the lipid present in the HDL particles. Two effector molecules mediate the inhibitory effect namely apolipoprotein A-I (apo A-I) and apolipoprotein A-II (apo A-II) (Brouillette C.G. and Anatharamaiah G.M. Biochem.Biophys. Acta. 1256: 103-129. 1995; Massey J.B., Pownall H.J. Biochem.Biophys Acta. 999 : 111-120. 198); these two molecules have different efficacy of inhibition.

Preferably, the high density lipoprotein or derivative thereof is a peptide or protein derivative of the sequence of apo A-I or apo A-II, or a peptide or protein derivative functionally homologous to the active portions of apo A-I or apo A-II.

Preferably, the high density lipoprotein is reconstituted HDL. The term "reconstituted HDL" means HDL composed of a lipid or lipids in association with at least one of the apolipoproteins of HDL. The components may be derived, for example, from blood, or produced by recombinant technology.

The medicament may be administered to a patient in any conventional manner. Preferably the medicament is administered intravenously. Preferably, the medicament is administered using saline as a vehicle.

Preferably the medicament is provided in a portable dispenser, for example, for use at the site of an accident.

According to the invention in another aspect there is provided a method of treatment of organ dysfunction following haemorrhagic shock in a human patient which comprises the step of administering to a patient reconstituted high density lipoprotein and/or a derivative thereof in pharmaceutically acceptable form.

The present invention will now be illustrated with reference to the attached drawings in which :

FIGURE 1 shows alterations in mean arterial blood pressure (MAP) in rats subjected to (i) the surgical procedure without causing a haemorrhage and treated with vehicle for HDL (SHAM, open diamonds, saline, 3mg/kg i.v. bolus; n=9) or with recHDL (SHAM - recHDL open square, 80mg/kg i.v. bolus injection, n=9) or (ii) haemorrhage for 1.5 h and upon resuscitation with the shed blood, control rats were treated with the vehicle (HS open circles, saline 3ml/kg i.v. bolus; n=10), recHDL (HS - recHDL filled squares, 80mg/kg i.v. bolus injection, n=9) or nHDL (HS - nHDL open triangle, 80 mg/kg i.v. bolus injection) [nHDL = native HDL; rHDL or recHDL is reconstituted HDC];

FIGURE 1A shows a Table of heart rate in beats per minute(bpm) in all experimental groups studied before the haemorhhage -1.5 h and 1, 2, 3 and 4 h after resuscitation, Group 1 (SHAM): Rats were subjected to the surgical procedure without causing a haemorrhage and treated with a vehicle for HDL (saline, 1ml/kg i.v. bolus followed by an infusion of 1.5 ml/kg/h i.v.; n=9); Group 2 (SHAM - recHDL): Rats

were subjected to the same surgical procedure as Group 1 but treated with recHDLs (reconstituted HDLs) (80mg/kg i.v. bolus injection followed by an infusion of saline 1.5 ml/kg/h i.v. n=4); Group 3 (HS): Rats were subjected to a haemorrhage for 1.5 h and upon resuscitation with the shed blood were given an infusion of 1.5 ml/kg/h i.v., n=9); Group 4 HS-recHDL: rats were subjected to the same procedure as group 3 but treated with recHDLs (80mg/kg i.v. bolus injection followed by an infusion of saline 1.5 ml/kg/h i.v.; n=9); and Group 5 (HS-nHDL) : rats were treated in the same way as Group 4, but were given nHDLs instead of recHDLs prior to resuscitation.

FIGURE 2 shows plasma levels of (A) urea, (B) creatine, (C) AST, (D) ALT, (F) creatinine kinase (CK) and (E) lipase in rats subjected to the surgical procedure and experiment 2 described below;

FIGURE 3 shows the effect of HDL infusion on histological sections from lung, gut and kidney following haemorrhagic shock;

FIGURE 4A shows the effect of HDLs on myeloperoxidase (MPO) levels in the lung, as a measure of neutrophil activation;

FIGURE 4B shows the effect of HDLs on MPO levels in the kidney;

FIGURE 5A shows the effect of HDLs on the level of malonialdehyde (MAD) in the lung;

FIGURE 5B shows the effect of HDLs on the level of

malonialdehyde in the kidney;

FIGURE 6 shows a graph of mean fluorescence intensity (dependent on inhibition of E-selectin), as described below;

FIGURE 7A shows a graph of mean fluorescence intensity of HUVEC (veinous EC) against concentration of lipoproteins apo A-I and apo A-II for experiment 3, below; and

FIGURE 7B shows a graph of mean fluorescence intensity of HuAEC (arterial EC) against concentration of lipoprotein apo A-I and apo A-II for experiment 3, below.

As a demonstration of an embodiment of the invention, Experiment 1 describes the effects of human high-density lipoprotein (HDL) on the circulatory failure and multiple organ dysfunction injury (MODS) such as renal dysfunction and liver dysfunction caused by severe haemorrhage and resuscitation in the anesthetised rat. It should be noted that this is a model of end stage organ failure generated by haemorrhagic shock, and is not known to be a result of endotoxin release.

All experiments described herein were performed in adherence to the National Institute of Health guidelines on the use of experimental animals and in adherence to *Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986,* published by HMSO, London.

Experiment 1

The study was carried out on Wistar rats (Tuck, Rayleigh, Essex, UK) weighing 250mg - 320g receiving a standard diet and water *ad libitum*. All animals were anaesthetised with thiopentone (120mg/kg i.p.) and anaesthesia was maintained by supplementary injections of thiopentone as required. The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket. The right femoral artery was catheterised and connected to a pressure transducer (Senso-Nor 840, Senso-Nor, Horten, Noway) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR). These were displayed on a data acquisition system (MacLab 8e, ADI Instruments, Hasting, UK) installed on an Apple Macintosh computer. The right carotid artery was cannulated to bleed the animals (see hereafter). The jugular vein was cannulated for the administration of drugs. The bladder was also cannulated to facilitate urine flow and to prevent the possibility of development of post-renal failure. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilise for 15 mins. Then, blood was withdrawn from the catheter placed in the carotid artery in order to achieve a fall in MAP to 50mmHg within 10 mins. Thereafter, MAP was maintained at 50mmHg for a total period of 90 mins by either withdrawal (during the compensation period) or re-injection of blood. It should be noted that in these experiments, the amount of shed blood re-injected during the 90 min period of haemorrhage did not exceed 10% of the total amount of the blood withdrawn. The amount of blood withdrawn for rats subjected to haemorrhage and treated with vehicle (control group) was 7.0 ± 0.4ml

(SD); the amount of blood withdrawn from rats subjected to haemorrhage and treated with HDL (treatment group) was 7.0 ± 0.3ml ($p>0.05$). At 90 min after initiation of haemorrhage, the shed blood and an equivalent volume of Ringer lactic solution was re-injected into the animal.

The results are shown in Figures 1, 1A (Table 1), 2, 3 and 4.

FIGURE 2 shows plasma levels of (A) urea, (B) creatinine, (C) AST, (D) ALT, (F) creatinine kinase (CK) and (E) lipase in rats subject to (i) SHAM the surgical procedure without causing a haemorrhage and treated with vehicle for HDL (sham+saline,saline, 3ml/kg i.v. bolus i.v.; n=9) or with reconstituted HDL (sham +recHDL; 80mg/kg i.v. bolus injection, n=4, or (ii) haemorrage for 1.5.h and upon resuscitation with the shed blood, control rats were treated with the vehicle (hs {+ saline}, saline 3ml/kg i.v. bolus n=9),reconstituted HDL (hs + recHDL, 80mg/kg i.v. bolus injection, n=9) or nHDL (HS - nHDLC). The administration regimes are detailed more fully in the text accompanying Fig.1A. Haemorrhage and resuscitation resulted in significant increases in the serum levels of urea and creatinine (n=9), as demonstrated by the increase in urea and creatine concentration between "sham" and hs (control). This renal dysfunction was attenuated by the administration (5 mins prior to resuscitation) of HDL (80mg/kg. i.v., n=9; p,0.05; ANOVA followed by Dunnett's test for multiple comparisons), as demonstrated by the concentration of urea and creatinine for "HS+ recHDL" and HS-nHDL. Similarly,

HDL attenuated the liver injury (as monitored by a rise in serum AST and ALT) - (C) and (D) - and the pancreatic injury (as measured by a rise in serum lipase - (E)) caused by haemorrhage and resuscitation. In contrast, recHDL and nHDL did not affect the delayed circulatory failure associated with haemorrhage and resuscitation (see Fig 1 and Fig.1A (Table 1)). Administration of recHDL to rats which were not subjected to haemorrhage did not result in the alterations in the serum levels of urea, creatinine, AST, ALT or lipase (n=4) and, hence, was not toxic at the dose used.

Organ dysfunction as measured by the degree of disruption of tissue architecture was reduced by treatment with HDLs.

Light microscopy. Organ (lung, kidney and small intestine) biopsies were taken at the end of Experiment 1 and fixed for one week in buffered formaldehyde solution (10% in PBS) at ambient temperature, dehydrated by graded ethanol, and embedded in Paraplast™ (Sherwood Medical, Mahwah, NJ, USA). Sections (7 µm thick) were deparafinised with xylene, and stained using either Van Gieson's Trichrome or Fuchsin, and examined using light microscopy (Dialux 22, Leitz).

Figure 3 shows photomicrographs of representative sections of lung (upper panels), small intestine (middled panels), and kidney (lower panels) from animals following haemorrhage for 90 min. and given vehicle (saline) along with shed blood at the beginning of resuscitation (A), nHDLCs (80mg/kg i.v. bolus injection) prior to resuscitation (B), or recHDLs (80mg/kg i.v. bolus injection prior to resuscitation (C).

Sections were visualised using Van Geison's trichrome stain. Original magnification x 100.

When compared to organs obtained from sham-operated rats, which had not been subjected to haemorrhage and resuscitation (data not shown), Fig.3 demonstrates that the lung (top panels), small intestine (middle panels) and kidney (bottom panels), when subjected to haemorrhage and resuscitation (A), show oedema with loss of normal tissue structure. In contrast, organs from animals which had received nHDLs (B) or recHDLs(C) prior to resuscitation showed no significant change in morphology, and were not significantly different from the sham-operated rats (not shown).

HDLs reduced neutrophil infiltration into lungs and kidneys following haemorrhage and resuscitation.

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation was determined as previously described [Anderson, B.O., Brown, J.M, Shanley, P.F., Benserd, D.D., and Harken, A.H. (1991 Marginating neutrophils are reversibly adherent to normal lung endothelium. *Surgery* 109.51-61). Samples of lung and kidney were obtained and weighed. Each piece of tissue was homogenised in a solution containing 0.5% hexadecyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0) and centrifuged for 30 min at 20,000 x g at 4°C. An aliquot of the supernatant was the allowed to react with a solution of tetra-methyl-benzidine

(1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. 1 mU of MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxidase per min at 37°C, and was expressed in mU per mg of wet tissue.

Figure 4 : Graph showing the effect of HDLs on myeloperoxidase (MPO) levels in (A) lung or (B) kidney, as a measure of neutrophil activation. Values represent mean and SEM, n=9; *p<0.05 when compared to haemorrhagic shock (HS).

The ability of HDLs to inhibit the expression of adhesion molecules in this model is strongly supported by the serum data and histology. We further investigated the ability of HDLs to inhibit neutrophil infiltration by measuring the MPO levels in lung (Figure 4A) and kidney (Figure 4B). When compared to tissues obtained from sham-operated rats, rats subjected to haemorrhage and resuscitation (solid bars) show an increase in tissue MPO activity. This was reduced in rats which had been treated with either nHDLs or recHDLs prior to resuscitation with shed blood.

HDLs reduced malondialdehyde levels in lungs and kidneys following haemorrhage and resuscitation.

Determination of malondialdehyde. Malondialdehyde (MDA) levels in the lung and kidney were determined as an indicator of lipid peroxidation. Tissues were homogenised in 1.15% KCI solution. An aliquot (100 µl) of the

homogenate was added to a reaction mixture containing 200 µl 8.1% SDS, 1500 µl 20% acetic acid (pH 3.5), 1500 µl 0.8% thiobarbituric acid and 700 µl distilled water. Samples were then boiled for 1 hour at 95°C and centrifuged 3,000 x g for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 650 nm.

Figure 5 : Bar graph showing the effect of HDLs on the level of malondialdehyde (MAD) in lung (A) and (B) kidney, as a measure of the anti-oxidant properties of HDLs. Values represent mean and SEM, n=9; *p,0.05 when compared to haemorrhagic shock (HS).

HDLs have been shown to have anti-oxidant properties. The ability of HDLs to influence the MDA levels in lung (Figure 5A) and kidney (Figure 5B) was investigated. When compared to tissues obtained from sham-operated rats, rats subjects to haemorrhage and resuscitation (solid bars) showed a marked increase in tissue MDA activity. This activity was reduced in rats which had been treated with either nHDLs or recHDLs prior to resuscitation [HS - nHDL, HS - recHDL].

In conclusion, administration of recHDL and nHDL attenuates the renal, liver and pancreatic dysfunction following haemorrhagic shock.

Experiment 2

This experiment demonstrates which components of the effective therapeutic agent (HDL) are responsible for protection against haemorrhagic shock; in this experiment,

the ability of native HDL to inhibit cytokine-induced adhesion molecule expression on endothelial cells is compared with the ability of lipid-free apo A-I protein or protein-free lipid vesicles.

Cell culture : Human umbilical vein-derived endothelial cells (HUVEC) and human umbilical-derived artery endothelial cells (HUAEC) (Cockerill G.W, Meyer G, Noack L.Vadas MA, Gamble J.R. Lab.Invest.71 : 497-509.1994) were grown on gelatin-coated tissue culture flasks (Costar, High Wycombe, Bucks, UK) in medium 199 with Earle's salts (Gibco, Paisley, Scotland) supplemented with 20% foetal calf serum (FCS) (Gibco, Australia), 20mM HEPES, 2mM glutamine, 1mM sodium pyruvate, non-essential amino acids, penicillin and streptomycin, 50µg/ml endothelial cell growth supplement (Sigma, Dorset, UK) and 50µg/ml heparin (normal growth medium).

Flow cytometry : Cells were plated at 1 x 10^5 cells/30 mm well and incubated overnight at 37°C in 5% CO_2. Confluent monolayer cultures were then incubated (at concentrations indicated) for 19 hours with either, phosphate buffered saline (PBS) (vehicle control), native HDL, free apo A-I, phospholipid vesicles or discoidal HDL prepared with only apo A-I or apo A-II. Following these treatments the cells were washed gently in complete medium and TNFα (Miles Scientific) was added at 10ng/ml. Cells were then stained at 4 hours post stimulation in the following manner. Cells were washed in serum free medium and 200µl anti-E-selectin (1,2B6) was added for 1 hour at 37°C. Cells were then washed

in phosphate buffered saline (PBS) containing 5% newborn calf serum, 0.02% sodium azide, and 200µl of fluorescein isothiocyanate-conjugated secondary antibody added for 1 hour at 37°C. Cells were then washed three times in PBS and trypsinised, then centrifuged to form a pellet. The pellet was then resuspended in 2.5% formaldehyde in PBS containing 2% glucose and 0.02% azide and analyzed in a Coulter Epics Profile II flow cytometer.

Figure 6 shows that neither free apo A-I nor unilamellar vesicles (SUV) were able to inhibit TNFα-induced expression of E-selectin. This suggests that Apo A-I, the most abundant apolipoprotein in HDL, must be in a lipid particle in order to mediate inhibition of cytokine-induced adhesion molecule expression in endothelial cells. Both umbilical-derived venous (HUVEC) and arterial (HUAEC) endothelial cells were able to support the dose-dependent inhibition of cytokine-induced E-selectin expression by HDL (as shown by the decrease in intensity with increase of apo AI HDL from 0.25 t0 1.0mg/ml).

The therapeutic action of HDL is afforded by the apolipoprotein presented in a lipid particle, and cannot be mimicked by the whole protein alone, or lipid alone.

Experiment 3

To determine the efficacy of reconstituted discoidal HDLs particles containing either of the most abundant apolipoproteins (apo A-I or apo A-II), a comparison of the ability of these particles to inhibit cytokine-induced

adhesion molecule expression on HUVEC and HUAEC was carried out.

Cell culture : Human umbilical vein-derived endothelial cells (HUVEC) and human umbilical-derived artery endothelial cells (HUAEC) (Cockerill et al., 19994) were grown on gelatin-coated tissue culture flasks (Costar, High Wycombe, Bucks, UK) in medium 199 with Earle's salts (Gibco, Paisley, Scotland), supplemented with 20% foetal calf serum (FCS) (Gibco, Australia), 20 mM HEPES, 2mM glutamine, 1mM sodium pyruvate, nonessential amino acids, penicillin and streptomycin, 50µg/ml endothelial cell growth supplement (Sigma, Dorset, UK) and 50µg/ml heparin (normal growth medium).

Flow cytometry : Cells were plated at 1 x 10^5 cells/30 mm well and incubated overnight at 37°C in 5% CO_2. Confluent monolayer cultures were then incubated (at concentrations indicated) for 19 hours with either reconstituted discoidal HDL prepared with only apo A-I or apo A-II. Following these treatments the cells were washed gently in complete medium and TNFα (Miles Scientific) was added at 10ng/ml. Cells were then stained at 4 hours post stimulation in the following manner. Cells were washed in serum free medium and 200µl anti-E-selectin (1.2B6) was added for 1 hour at 37°C. Cells were then washed in phosphate buffered saline (PBS) containing 5% newborn calf serum, 0.02% sodium azide, and 200µl of fluorescein isothiocyanate-conjugated secondary antibody added for 1 hour at 37°C. Cells were then washed three time in PBS and trypsinised. The pellet was then

resuspended in 2.5% formaldehyde in PBS containing 2% glucose and 0.02% azide and analyzed in a Coulter Epics Profile II flow cytometer.

Preparation of Reconstituted HDL Particles : Discoidal reconstituted A-I HDLs were prepared by the cholate dialysis method from egg yolk phosphatidylcholine, unesterified cholesterol, and apo A-I/apo A-II (Matz CE, Jonas A. Micellar complexes of human apolipoprotein A-I with phosphatidycholines and cholesterol prepared from cholate-lipid dispersion. *J.Biol.Chem.1982; 257; 4535-4540*). Egg yolk phosphatidycholine, unesterified cholesterol and sodium cholate were obtained from Sigma and used without further purification. Particle size was measured by nondenaturing gradient gel electrophoresis, and concentration of apo A-I and apo A-II was measured immunoturbidimetrically.

Results : Discoidal reconstituted HDL particles containing either apo A-I (open squares) or apo A-II (closed squares), as the sole protein, were able to inhibit TNFα-induced expression of both arterial and venous endothelial cells VCAM-1. Figure 7a (HUVEC) and 7b (HuAEC) show reconstituted HDL containing apo A-I, as the sole proteins, having a t1/2 max of approximately 3 µMolar, whilst reconstituted HDL containing apo A-II as the sole protein has a give five-fold greater t1/2 max of 15 µMolar.

Conclusion : The therapeutic action of HDL can be mimicked using either apo A-I or apo A-II in reconstituted lipoprotein particle.

C L A I M S :

1. Use of high density lipoprotein and/or a derivative thereof in the manufacture of a medicament for the prevention or treatment of organ dysfunction following haemorrhagic shock.

2. Use according to claim 1 where the medicament is for the treatment of end-stage organ injury or failure.

3. Use according to claim 1 or 2 where the high density lipoprotein or derivative thereof is a peptide or protein derivative of the sequence of apo A-I or apo A-II, or a peptide or protein derivative functionally homologous to the active portions of apo A-I or apo A-II.

4. Use according to claim 1 or 2 where the high density lipoprotein is reconstituted HDL.

5. Use according to claim 1 or 2 where the high density lipoprotein is native HDL.

6. A method of treatment of organ dysfunction following haemorrhagic shock in a human patient which comprises the step of administering to the patient reconstituted high density lipoprotein and/or a derivative thereof in pharmaceutically acceptable form.

HIGH DENSITY LIPOPROTEIN AGAINST ORGAN DYSFUNCTION FOLLOWING HAEMORRHAGIC SHOCK

ABSTRACT

Use of high density lipoprotein and/or derivatives in the manufacture of a medicament for the prevention or treatment of organ dysfunction following ischaemia and reperfusion injury. In particular, the medicament may be for the treatment of end stage organ injury or failure.

FIGURE 1

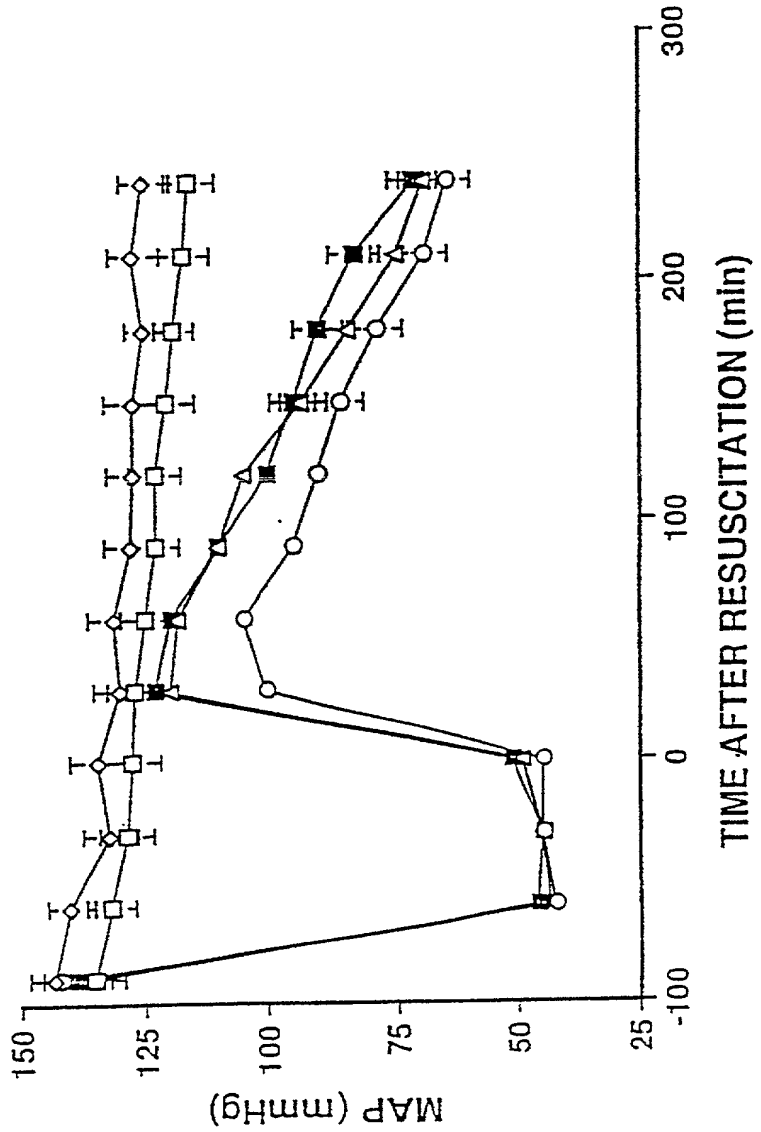


FIGURE 1A Heart rate (beats per min) in all experimental groups before hemorrhage (Baseline) and 1,2,3, and 4 h after resuscitation.

Group	n	Baseline	Resuscitation Time (h)			
			1	2	3	4
1. SHAM	9	381 ± 15	369 ± 11	385 ± 13	384 ± 13	377 ±
2. SHAM-recHDL	4	416 ± 10	387 ± 12	376 ± 8	393 ± 6	380 ±
3. HS	9	386 ± 11	417 ± 10	423 ± 20	398 ± 24	372 ±
4. HS-recHDL	9	364 ± 13	386 ± 13	407 ± 10	391 ± 14	378 ±
5. HS-nHDL	7	388 ± 11	381 ± 15	369 ± 8	356 ± 12	340 ±

Group 1: Rats were subjected to the surgical procedure without causing a hemorrhage and treated with vehicle for HDLs (saline, 1ml/kg i.v. bolus followed by an infusion of 1.5 ml/kg/h i.v.; n=9); Group 2: Rats were subjected to the same surgical procedure as group 1 but were treated with recHDLs (80mg/kg i.v. bolus injection, followed by an infusion of saline 1.5ml/kg/h i.v., n=4); Group 3: Rats were subjected to a hemorrhage for 1.5 h and upon resuscitation with the shed blood were given an infusion of saline (1.5ml/kg/h i.v.., n=9); Group 4: Rats were subjected to the same procedure as Group 3 but treated with recHDLs (80mg/kg i.v.bolus injection, followed by an infusion of saline 1.5ml./kg/h i.v., n=9); Group 5: Rats were treated in the same way as Group 4, but were given nHDLs instead of recHDLs prior to resuscitation. There were no significant differences in the hear rates between any of the experimental groups.

FIGURE 2A

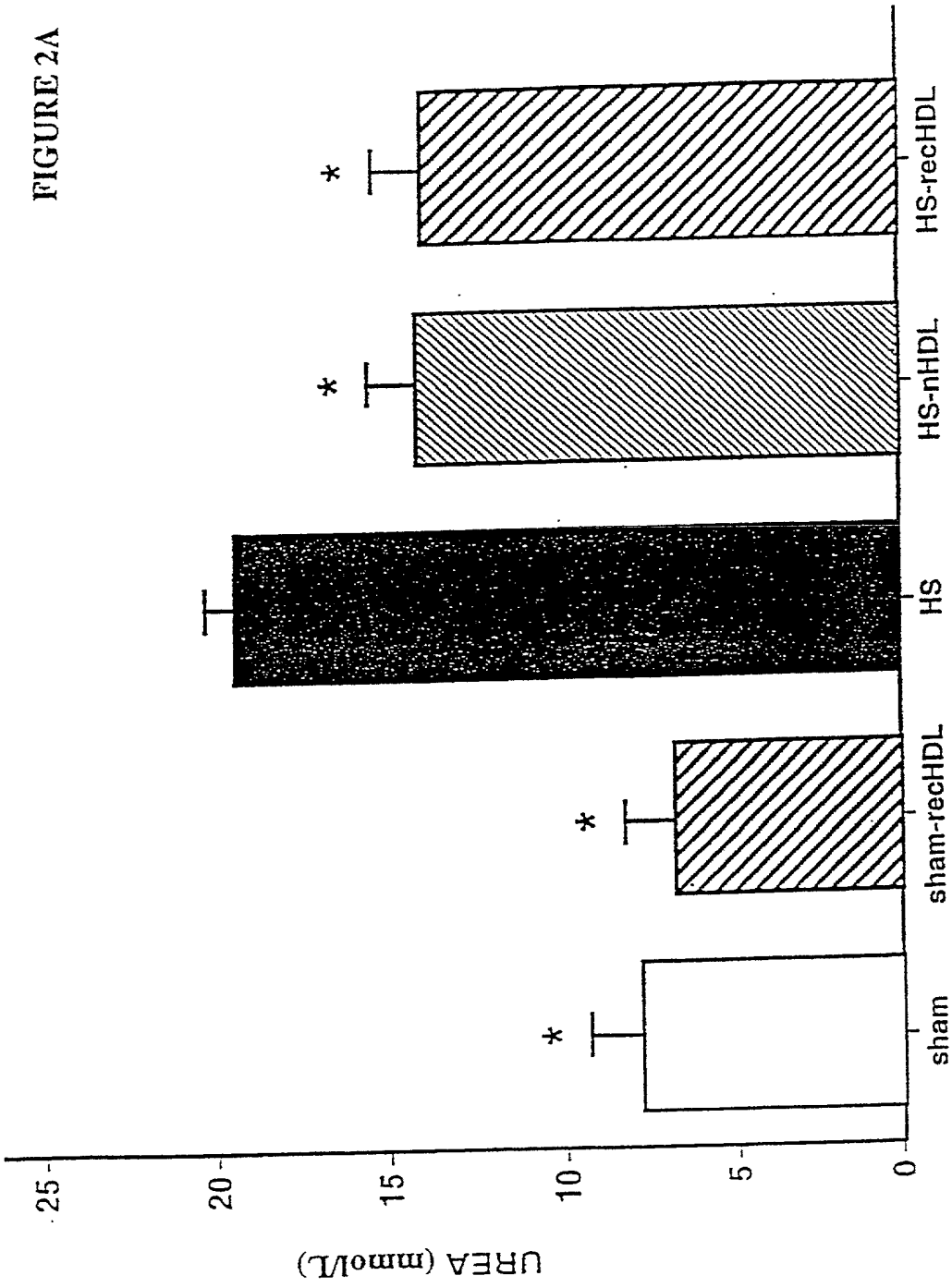


FIGURE 2B

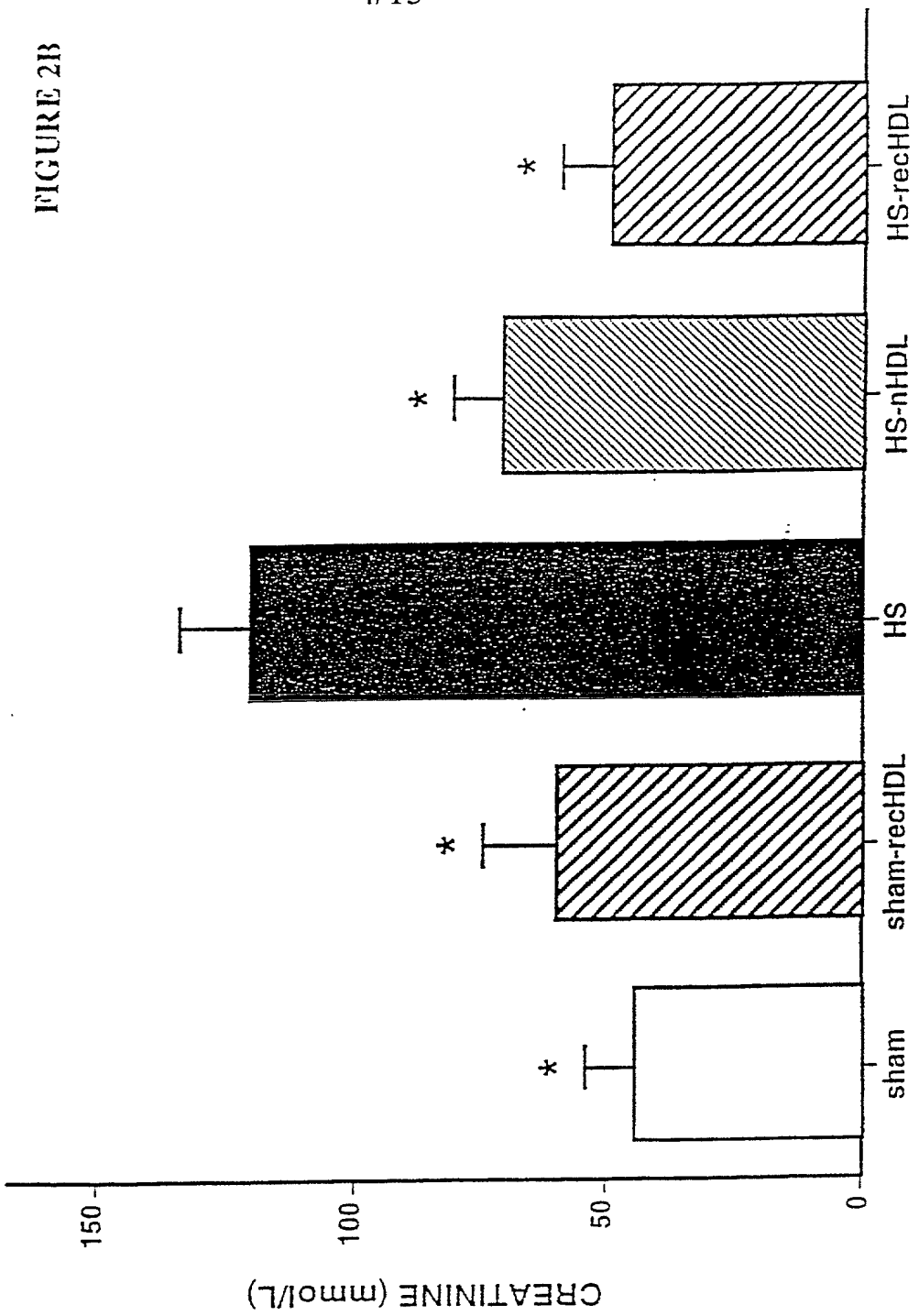

CREATININE (mmol/L)
150
100
50
0
sham
sham-recHDL
HS
HS-nHDL
HS-recHDL
*
*
*
*
*

FIGURE 2C

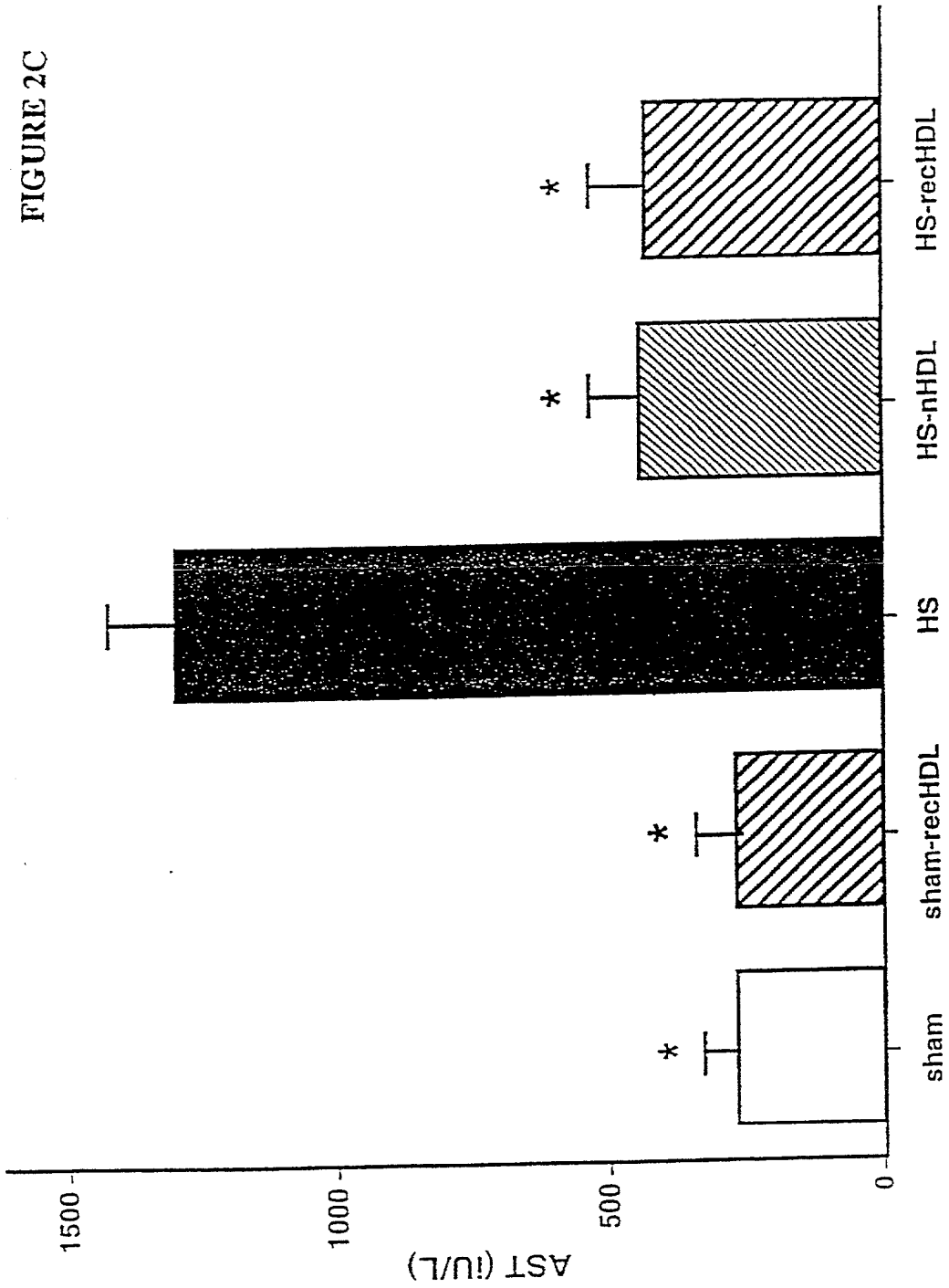


FIGURE 2D

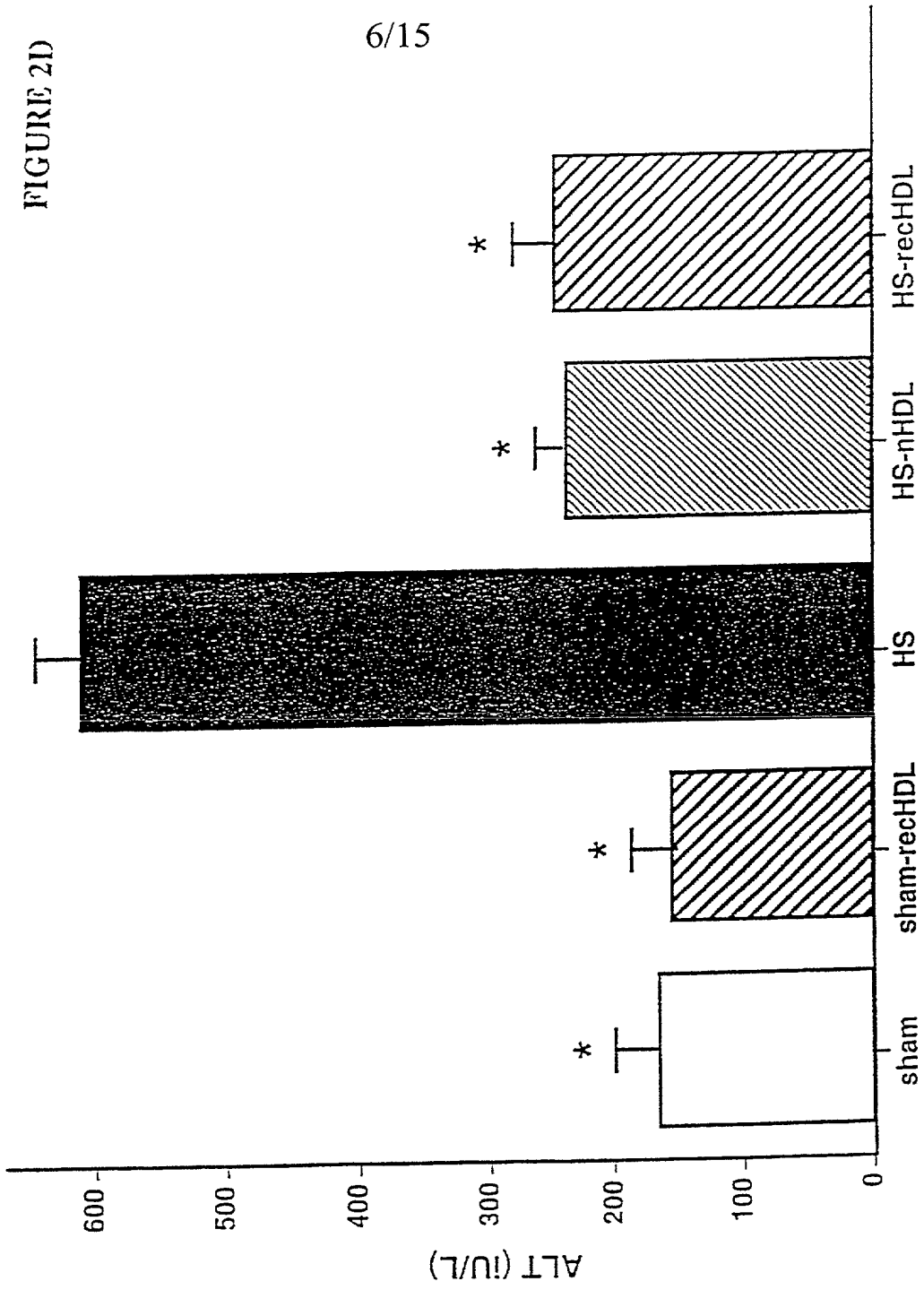

ALT (iU/L)
0
100
200
300
400
500
600
sham
sham-recHDL
HS
HS-nHDL
HS-recHDL
*
*
*
*
*

FIGURE 2E

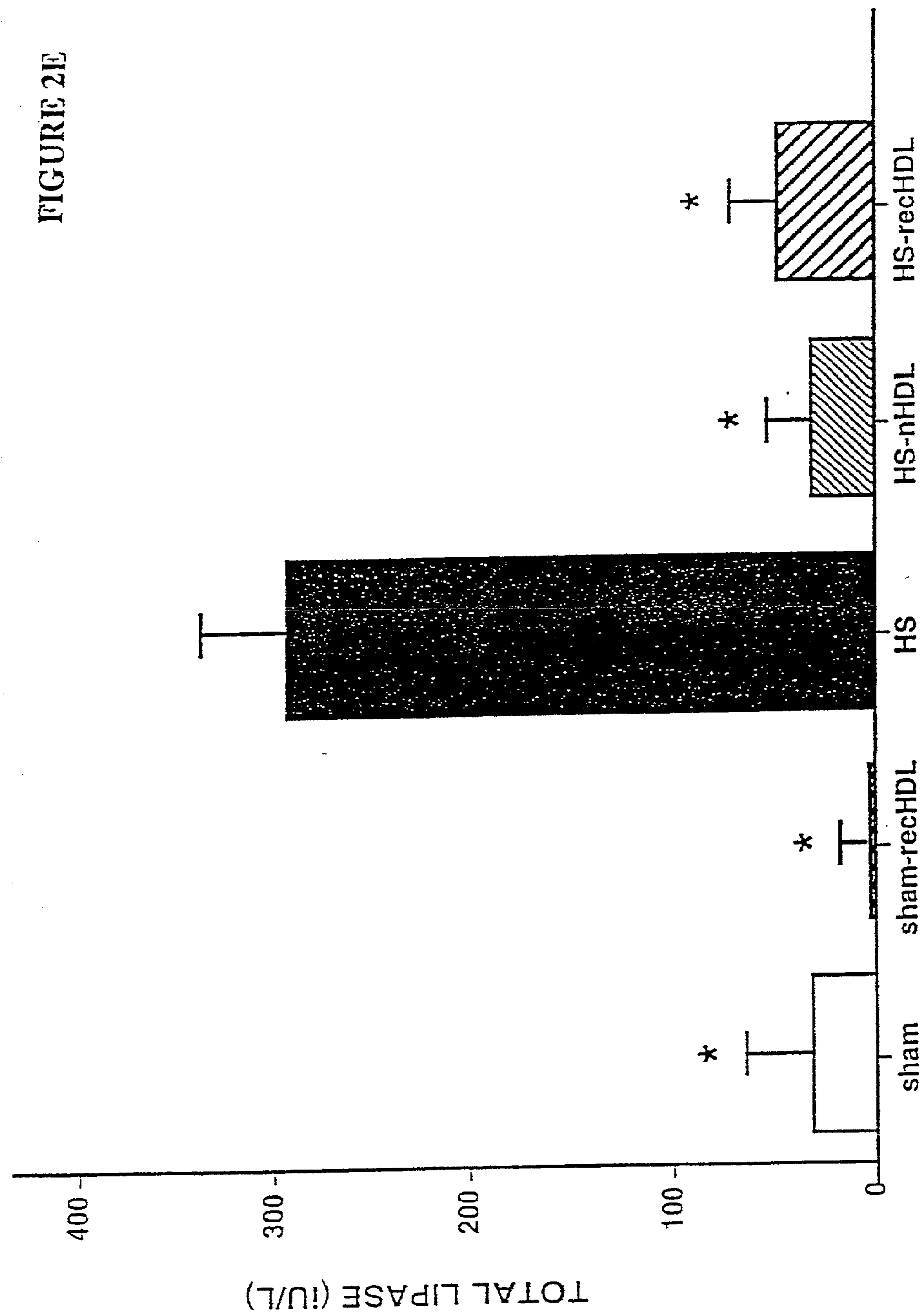


FIGURE 2F

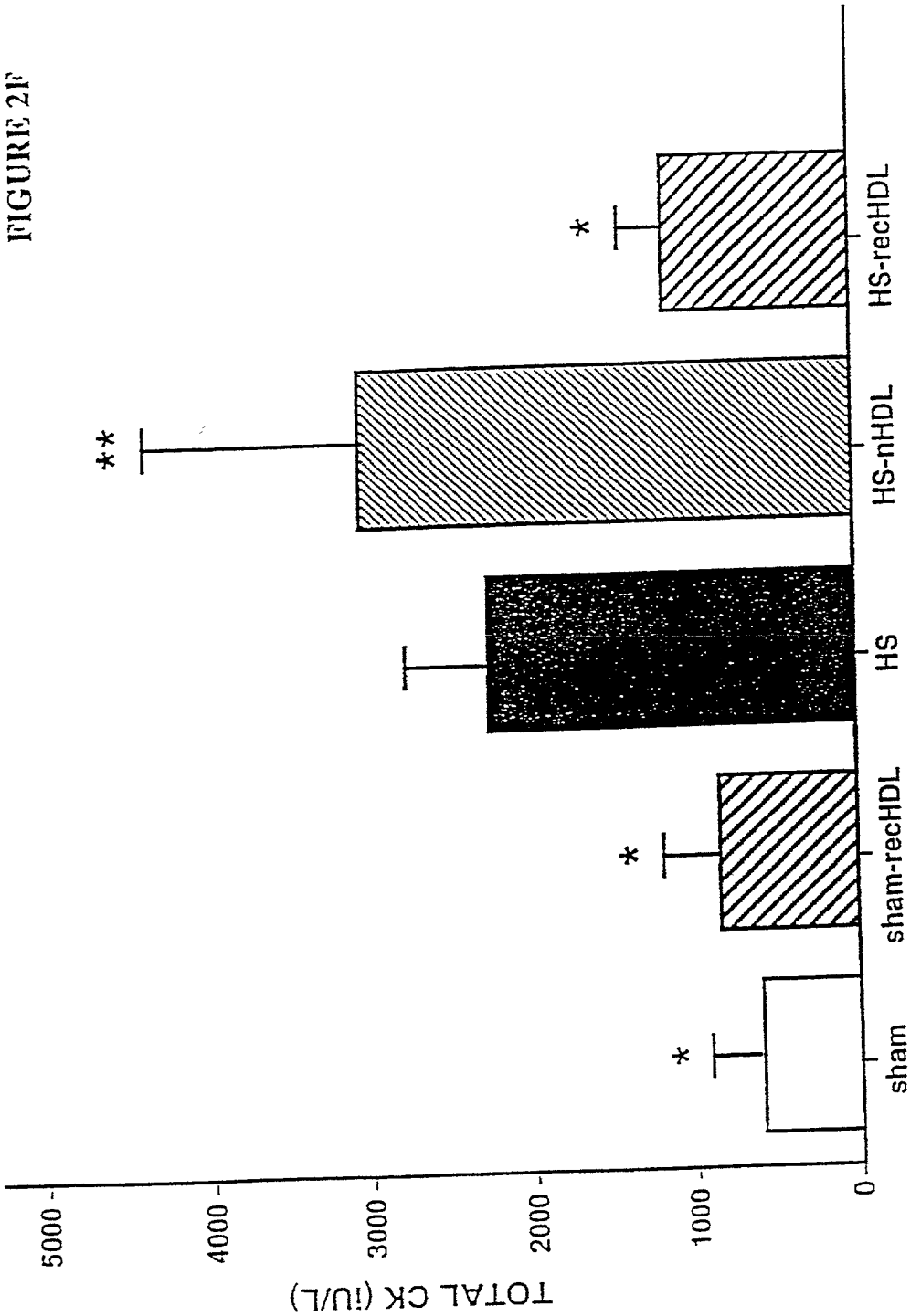


FIGURE 3

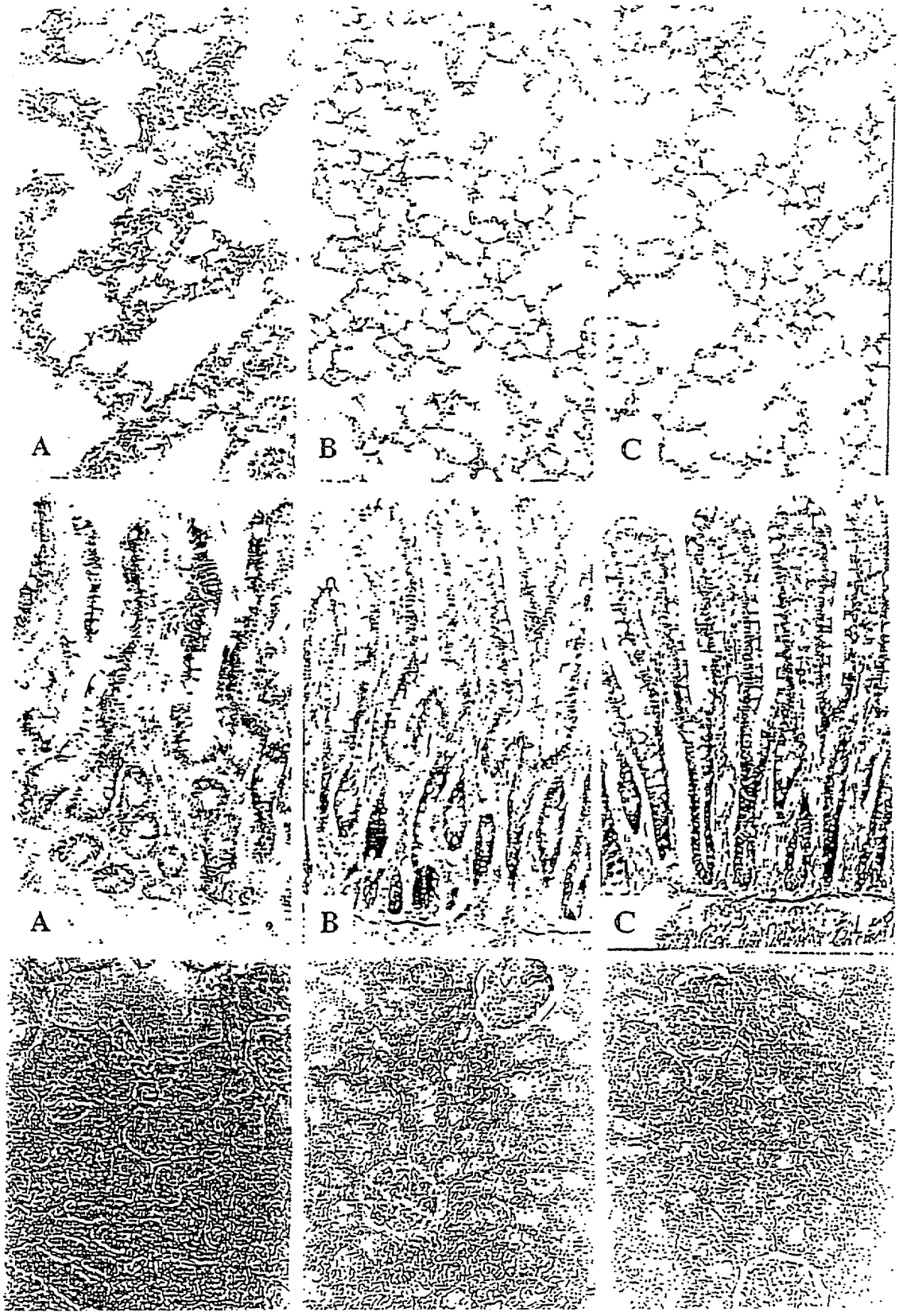


FIGURE 4A

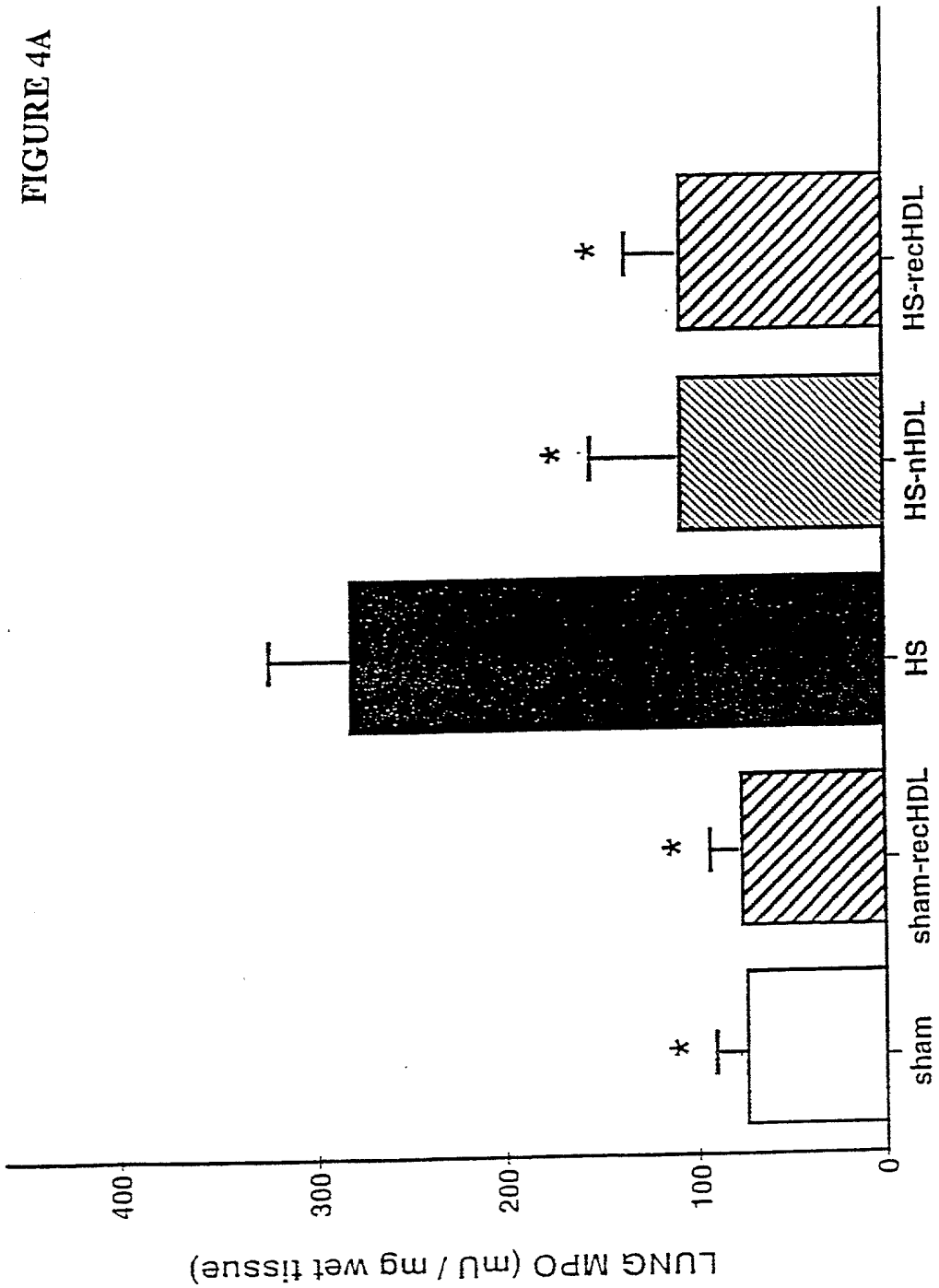


FIGURE 4B

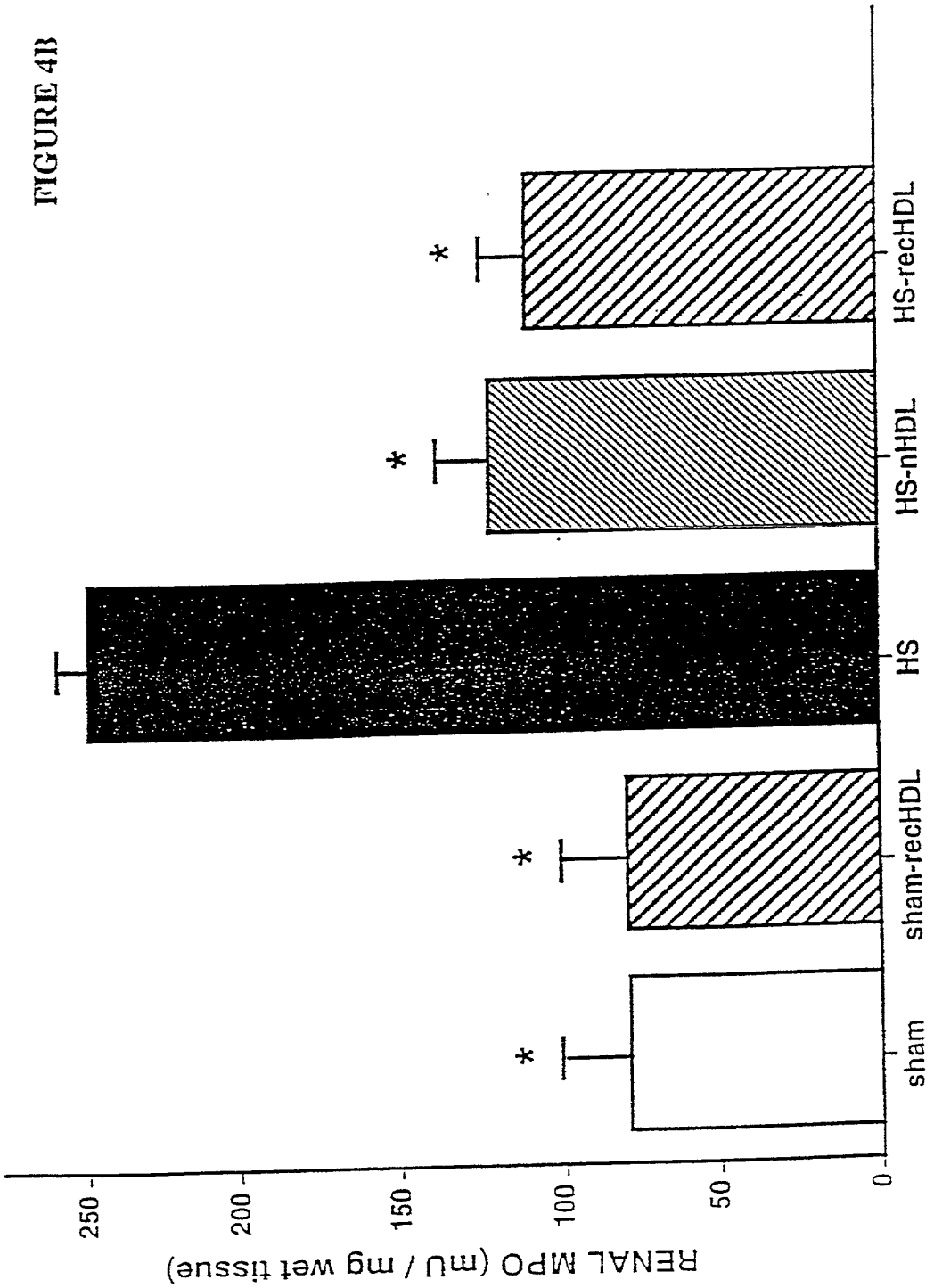
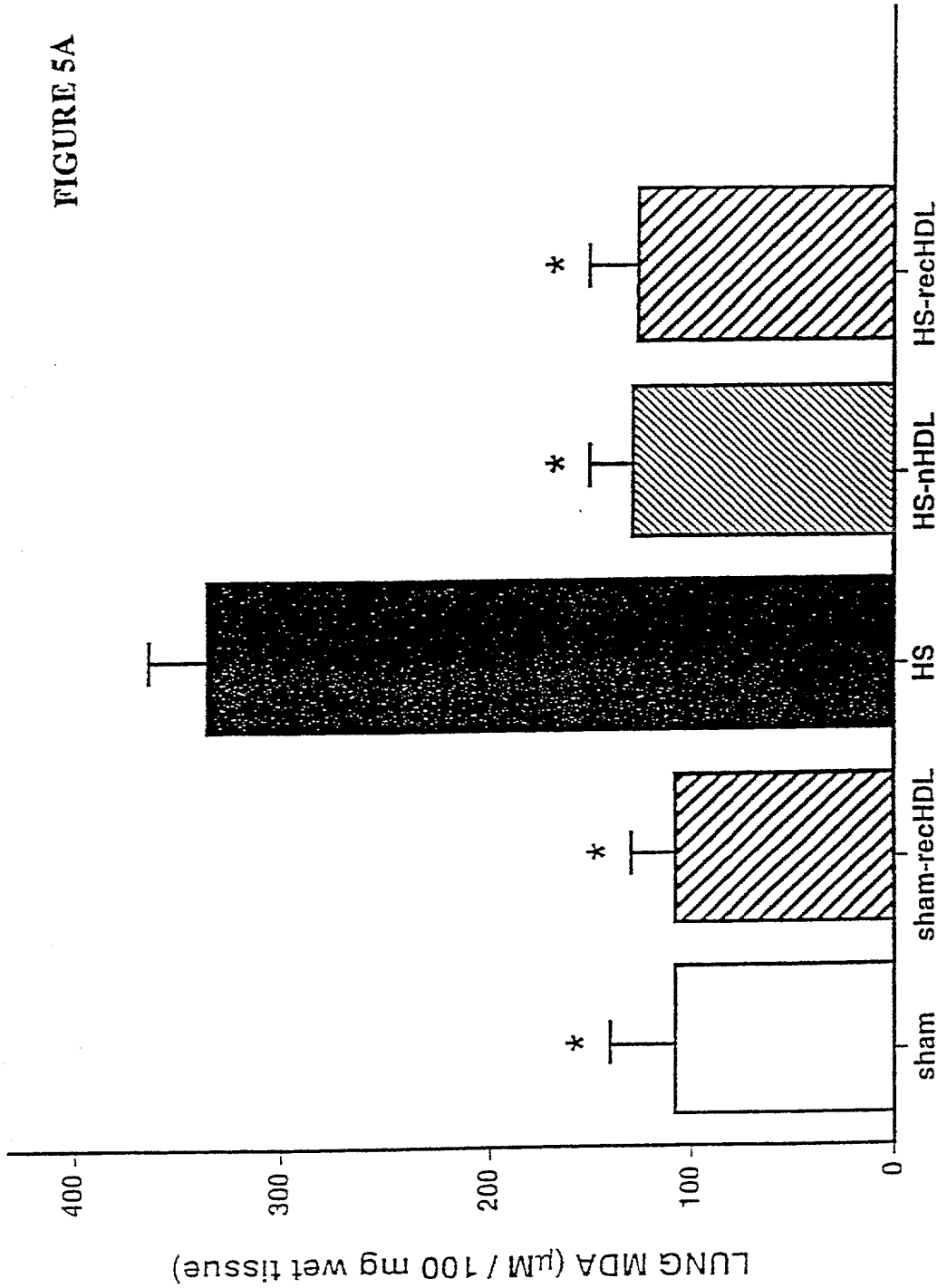


FIGURE 5A


LUNG MDA (μM / 100 mg wet tissue)
400
300
200
100
0
sham
sham-recHDL
HS
HS-nHDL
HS-recHDL
*
*
*
*

10/069157

FIGURE 5B

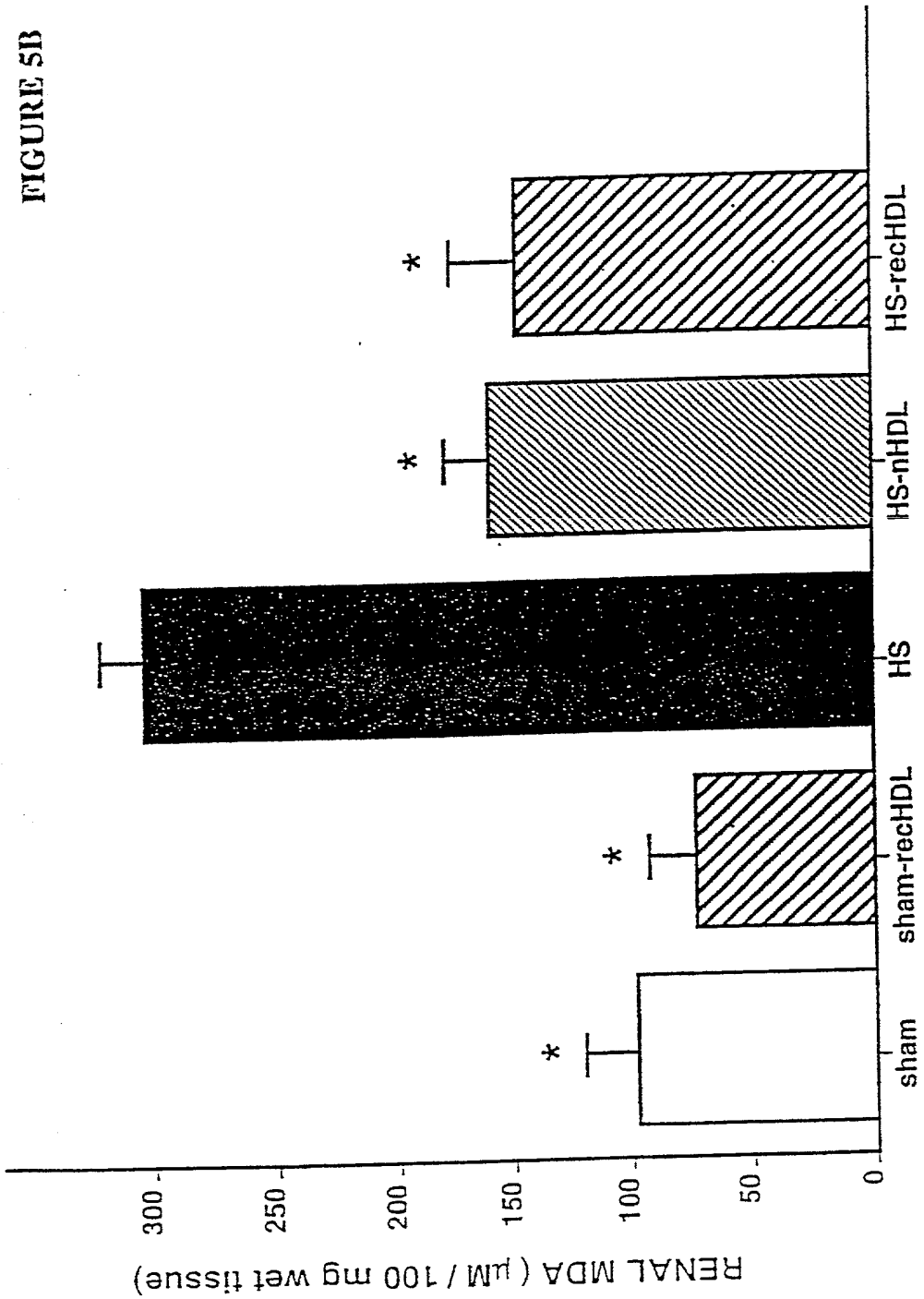


FIGURE 6

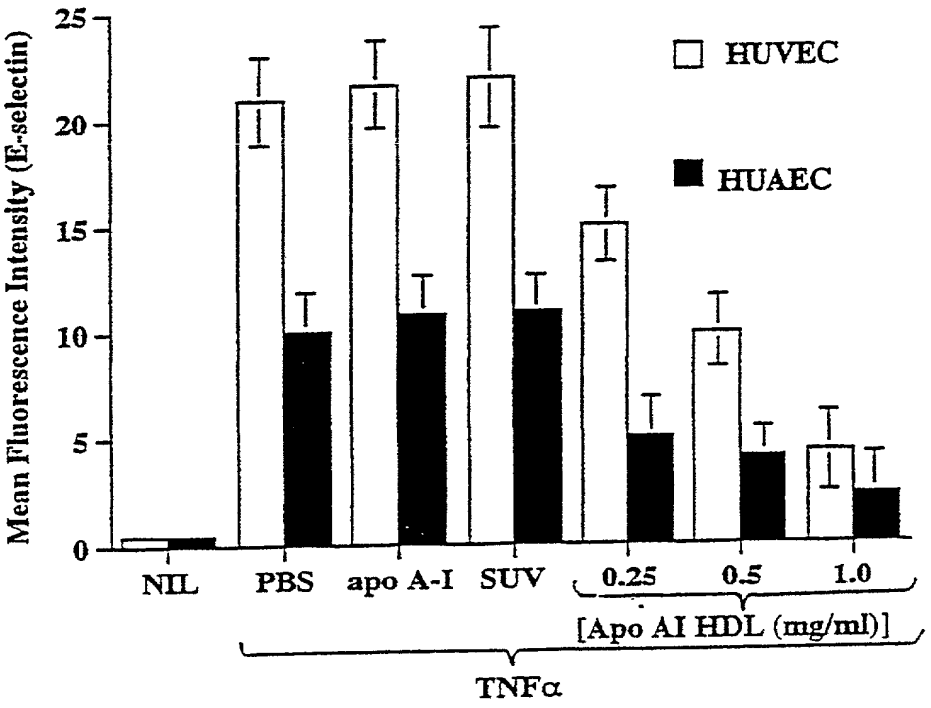


FIGURE 7A

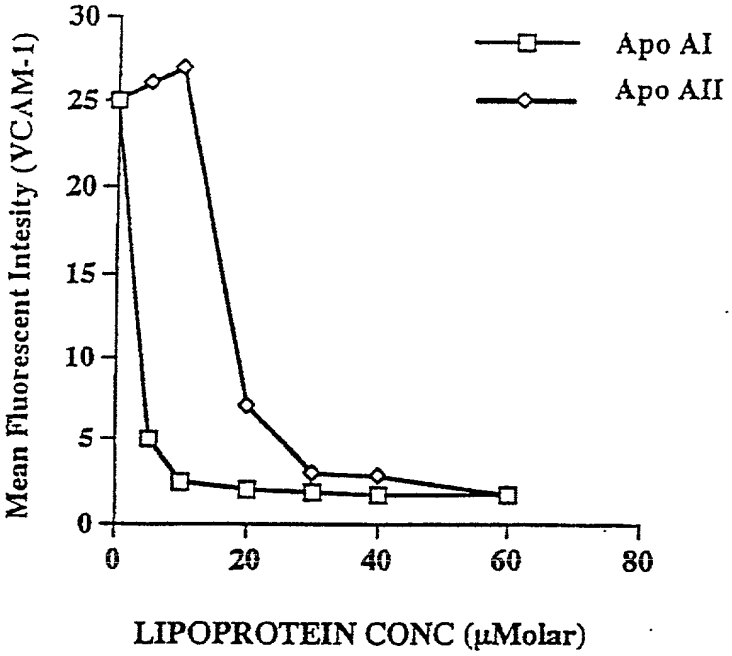
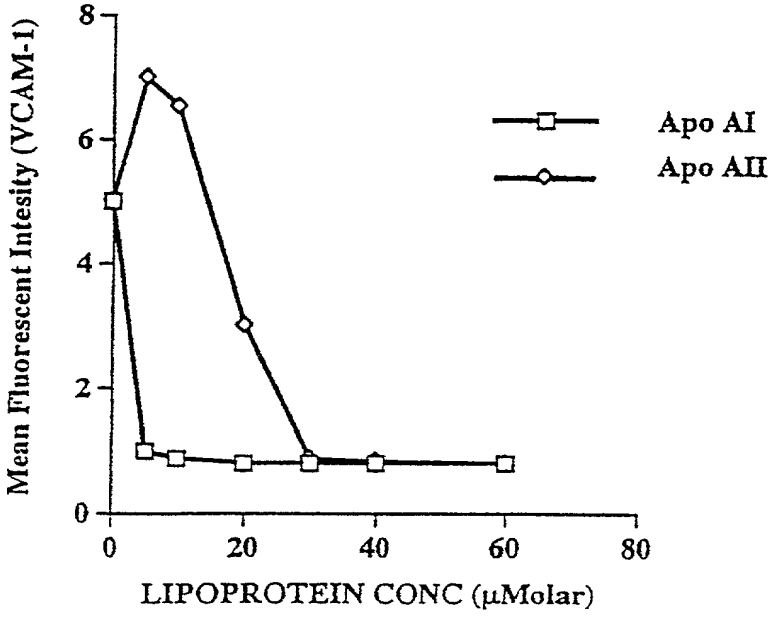


FIGURE 7B



PTO/SB/01(12/97)

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled HIGH DENSITY LIPOPROTEIN AGAINST ORGAN DYSFUNCTION FOLLOWING HAEMORRHAGIC SHOCK, the specification of which

☐ is attached hereto

or

☒ was filed on August 16, 2000 as United States Application No. or PCT International Application No. PCT/GB00/03182 and was amended on _____. (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed	Cert. Copy Attached	
GB 9919713.9 (Number)	Great Britain (Country)	19/August/1999 (Day/Month/Year)	☐	Yes	No
(Number)	(Country)	(Day/Month/Year)	☐	Yes	No
(Number)	(Country)	(Day/Month/Year)	☐	Yes	No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

(Application Serial No.) (Filing Date)

(Application Serial No.) (Filing Date)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365(c) of any PCT International application designating the United States, listed

below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Parent Patent Number) (if applicable)
(Application Serial No.)	(Filing Date)	(Parent Patent Number) (if applicable)

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Full name of fifth joint inventor, if any _____

Inventor's signature _____ Date _____

Residence _____

Citizenship _____

Post Office Address _____

Full name of sixth joint inventor, if any _____

Inventor's signature _____ Date _____

Residence _____

Citizenship _____

Post Office Address _____

[ ] Additional inventors are being named on the _____ supplemental additional inventor(s) sheet(s).

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